# 10 BENZODIAZEPINE QUANTITATION AND CONFIRMATION BY GC/ECD AND GCMS

## 10.1 Summary

10.1.1 Benzodiazepines are extracted from biological samples by making the samples basic with saturated borate buffer and extracting with toluene/hexane/isoamyl alcohol (THIA). An aliquot of the organic extract is injected onto a GC equipped with an electron capture detector (or MSD) for quantitative analyses. The remainder of the organic extract is washed, concentrated (derivatized, if necessary) and injected onto a GCMS for confirmation. If lorazepam and/or temazepam are identified or suspected, the samples must be re-extracted with toluene and derivatized with MTBSTFA to form the t-butyldimethylsilyl derivatives which are analyzed by GCMS.

## 10.2 Specimen Requirements

10.2.1 1 mL whole blood or other biological fluids and tissue homogenates.

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## 10.3 Reagents and Standards

10.3.1 Diazepam, 1 mg/mL

10.3.2 Nordiazepam, 1 mg/mL

10.3.3 Alprazolam, 1 mg/mL

10.3.4 Clonazepam, 1 mg/mL

10.3.5 N-desalkylflurazepam, 1 mg/mL

10.3.6 Chlordiazepoxide, 1 mg/mL

10.3.7 Lorazepam, 1 mg/mL

10.3.8 Temazepam, 1 mg/mL

10.3.9 Prazepam, 1 mg/mL (internal standard)

10.3.10 Oxazepam-$d_5$, 0.05 mg/mL (internal standard)

10.3.11 Sodium tetraborate decahydrate

10.3.12 Hexane

10.3.13 Isoamyl alcohol

10.3.14 Methanol

10.3.15 Dry toluene (toluene saturated with sodium sulfite)

10.3.16 Acetonitrile

10.3.17 N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA)

**10.4 Solutions, Internal Standard, Calibrators, Controls**

10.4.1 Saturated borate buffer solution. Add sodium tetraborate decahydrate to $dH_2O$ until no more dissolves after shaking vigorously.

10.4.2 Toluene:Hexane:Isoamyl Alcohol (THIA) ( 78:20:2, v:v:v) Mix 78 mL toluene, 20 mL hexane and 2 mL isoamyl alcohol.

10.4.3 Drug stock solutions:

10.4.3.1 If 1 mg/mL commercially prepared stock solutions are not available, prepare 1 mg/mL solutions from powders. Weigh 10 mg of the free drug, transfer to a 10 mL volumetric flask and QS to volume with methanol. Note: If using the salt form, determine the amount of the salt needed to equal 10 mg of the free drug, and weigh this amount. Stock solutions are stored capped in a refrigerator and are stable for 2 years. Chlordiazepoxide is light sensitive and should be made each time a chlordiazepoxide calibration curve is generated (curve is only generated when quantitating a case containing chlordiazepoxide).

10.4.4 Working Standard Solution A (10 µg/mL diazepam and nordiazepam; 2 µg/mL clonazepam and alprazolam).

10.4.4.1 Add 100 µL each of diazepam and nordiazepam 1 mg/mL stock solutions and 20 µL each of clonazepam and alprazolam 1 mg/mL stock solutions to a 10 mL volumetric flask. QS to volume with methanol.

10.4.5 Working Standard Solution B (20 µg/mL chlordiazepoxide and 2 µg/mL n-desalkylflurazepam)

10.4.5.1 Add 200 µL of chloridazepoxide 1 mg/mL stock solution and 20 µL of n-desalkylflurazepam 1 mg/mL stock solution to a 10 mL volumetric flask. QS to volume with methanol.

10.4.6 Working Standard Solution C (50 µg/mL temazepam and lorazepam)

10.4.6.1 Add 500 µL each of temazepam and lorazepam 1 mg/mL stock solutions to a 10 mL volumetric flask. QS to volume with $dH_2O$.

10.4.7 Internal Standard working solution (20 µg/mL prazepam)

10.4.7.1 Add 200 µL of prazepam 1 mg/mL stock solution to a 10 mL volumetric flask. QS to volume with methanol.

10.4.8 Internal Standard working solution for lorazepam and temazepam (5 µg/mL oxazepam-$d_5$)

10.4.8.1 Add 1 mL of oxazepam-$d_5$ 0.05 mg/mL standard solution to a 10 mL volumetric flask. QS to volume with methanol.

10.4.9 Blood calibrators, standards, and controls preparation:

10.4.9.1 To prepare the following calibration curve, pipet the following volumes of working standard solution A into appropriately labeled 13 x 100 mm screw cap test tubes

| Amount of working standard (µL) | Final concentration of nordiazepam and diazepam (mg/L) | Final concentration of clonazepam and alprazolam (mg/L) |
|---|---|---|
| 100 | 1.0 | 0.2 |
| 75 | 0.75 | 0.15 |
| 50 | 0.5 | 0.1 |
| 20 | 0.2 | 0.04 |
| 10 | 0.1 | 0.02 |

10.4.9.1.1 Evaporate standards to dryness under nitrogen. Add 1 mL blank blood to each tube and vortex.

10.4.9.2 Standards B and C contain less frequently encountered benzodiazepines (chlordiazepoxide, n-desalkylflurazepam, lorazepam and temazepam). During routine benzodiazepine analyses, standards B and C may be analyzed for retention times. If any of the drugs are present or suspected, the analysis is repeated with a full calibration curve.

10.4.9.2.1 For routine analyses, pipet 100µL of working Standard Solution B into an appropriately labeled 13 x 100 mm labeled screw-cap test tube. Evaporate to dryness under nitrogen. Add 1 mL blank blood for a final concentration of 2 mg/L chlordiazepoxide and 0.2 mg/L n-desalkylflurazepam.

10.4.9.2.2 If a full calibration curve is required, pipet the following volumes of working standard solution B into appropriately labeled 13 x 100 mm screw cap test tubes.

| Amount of working standard (µL) | Final concentration of chlordiazepoxide (mg/L) | Final concentration of n-desalkylflurazepam (mg/L) |
|---|---|---|
| 250 | 5.0 | 0.5 |
| 100 | 2.0 | 0.2 |
| 50 | 1.0 | 0.1 |
| 25 | 0.5 | 0.05 |
| 10 | 0.2 | 0.02 |

10.4.9.2.3 For routine analyses, pipet 100µL of working Standard Solution C into an appropriately labeled 13 x 100 mm labeled screw-cap test tube. Evaporate to dryness under nitrogen. Add 1 mL blank blood for a final concentration of 1 mg/L temazepam and lorazepam.

10.4.9.2.4 If a full calibration curve is required, pipet the following volumes of working standard solution C into appropriately labeled 13 x 100 mm screw cap test tubes.

| Amount of working standard (µL) | Final concentration of lorazepam and temazepam (mg/L) |
|---|---|
| 200 | 1.0 |
| 100 | 0.5 |
| 40 | 0.20 |
| 20 | 0.1 |
| 10 | 0.05 |
| 4 | 0.02 |

10.4.9.2.5 Evaporate standards to dryness under nitrogen. Add 1 mL blank blood to each tube.

10.4.9.3 Controls

10.4.9.3.1 Negative control. Blood bank blood (or comparable) determined not to contain benzodiazepines.

10.4.9.3.2 Positive control. Control may be from a external source or prepared in house using drugs from different manufacturers, lot numbers or prepared by a chemist different than the individual performing the extraction.

**10.5 Apparatus**

10.5.1 Agilent GC/MSD, Chemstation software, compatible computer & printer

10.5.2 Agilent GC with Electron Capture Detector, Chemstation software, compatible computer & printer

10.5.3 Test tubes, 13 x 100 mm round bottom, screw cap tubes, borosilicate glass with Teflon caps

10.5.4 Test tubes, 16 x 114 mm (10 mL) glass tubes, conical bottom

10.5.5 Centrifuge capable of 2,000 – 3,000 rpm

10.5.6 Vortex mixer

10.5.7 Evaporator/concentrator

10.5.8 GC autosampler vials and inserts

10.5.9 Test tube rotator

10.5.10 GC/ECD parameters. Instrument conditions may be changed to permit improved performance.

10.5.10.1 Oven program.

- Equilibration time: 0.50 minutes
- Initial temp: 120° C
- Initial time: 0.8 minutes
- Ramp: 17° C/min
- Final Temp: 280° C
- Final Time: 7 minutes
- Run Time: 17 minutes

10.5.10.2 Inlet.

- Mode: Splitless
- Temperature: 250° C
- Constant pressure: 17 psi
- Purge flow: 20 mL/min
- Total flow: 25 mL/min
- Injection volume: 2.0 µL

10.5.10.3 Detector.

- Temperature: 350° C
- Makeup gas: Argon methane 5%
- Mode: Constant column + makeup flow
- Combined flow: 20.0 mL/min
- Injection volume: 1.0 µL
- Makeup flow: On

10.5.10.4 Column: HP-5 25 m x 0.25 mm x 0.25µm.

10.5.11 GCMSD parameters. Instrument conditions may be changed to permit improved performance.

10.5.11.1 Acquisition Mode: Scan (50 – 550 amu)

10.5.11.2 Column: HP 5MS 25 m x 0.25 mm x 0.25 µm

10.5.11.3 Detector Temperature: 280° C

10.5.11.3.1 Oven Program

- Equilibration time: 0.50 minutes
- Initial temp: 110° C
- Initial time: 1 minutes
- Ramp: 10° C/min
- Final Temp: 290° C
- Final Time: 9 minutes
- Run Time: 28 minutes

10.5.11.3.2 Inlet

- Mode: Splitless
- Temperature: 270° C
- Injection volume: 2.0 µL
- Purge Time: ON at 1.0 minute

**10.6 Procedure**

10.6.1 Label clean 13 x 100 mm screw cap tubes accordingly, negative, calibrators, control(s) and case sample IDs.

10.6.2 Prepare calibrators and controls.

10.6.3 Pipet 1 mL of each case sample into appropriately labeled tubes.

10.6.4 Add 30 µL internal standard working stock solution (20 µg/mL prazepam) to each tube.

10.6.5 Add 1 mL saturated borate buffer and 3 mL extract solvent (78:20:2 THIA) to each tube.

10.6.6 Cap and rotate tubes for 30 minutes.

10.6.7 Centrifuge at approx 2500 rpm for 15 minutes. Transfer a small amount of organic upper layer (THIA) to GC autosampler vial. Crimp autosampler vials and set aside for GC/ECD analysis.

10.6.8 Transfer remaining organic upper layer (THIA) to appropriately labeled conical bottom test tubes. Cap tubes and store in refrigerator for GC/MS confirmation of any positive samples (10.6.10).

10.6.9 Transfer GC autosampler vials to GC/ECD and analyze. Calculate the concentrations by interpolation of a linear plot of the response curve based on peak height or area. Identify any cases that possibly contain lorazepam, temazepam, chlordiazepoxide or n-desalkylflurazepam (based on peaks with relative retention times similar to standards B and C).

10.6.10 For cases in which diazepam, nordiazepam, clonazepam and/or alprazolam were detected by GC/ECD, remove the remaining THIA extracts from the refrigerator. Evaporate THIA extracts to dryness under nitrogen at $55^0$ C or less.

10.6.10.1 Cool samples to room temperature. Add 0.5 mL acetonitrile and 2 mL hexane to each sample.

10.6.10.2 Vortex each sample for 30 seconds at the highest setting.

10.6.10.3 Aspirate off top (organic) layer and discard. Evaporate remaining lower (acetonitrile) layer under nitrogen at 55° C or less.

10.6.10.4 Reconstitute samples with 30 µL 78:20:2 THIA and transfer to GC autosampler vials. Inject 1-2 µL of each sample onto GC equipped with MSD for confirmation.

10.6.10.5 For cases in which clonazepam was detected or suspected, samples must be derivatized with MTBSTFA for MSD confirmation . Follow procedure up to 10.6.10.3, but then reconstitute with 30 µL MTBSTFA and heat at 85° C for up to 1 hour. Cool to room temperature and inject 1-2 µL onto GC equipped with MSD.

10.6.11 For cases in which lorazepam and/or temazepam were detected or suspected, samples must be re-analyzed along with full calibration curve for lorazepam and temazepam.

10.6.11.1 Label clean 13 x 100 mm screw cap tubes accordingly, negative, calibrators, control(s) and case sample IDs.

10.6.11.2 Prepare calibrators and controls.

10.6.11.3 Pipet 1 mL of each case sample into appropriately labeled tubes.

10.6.11.4 Add 50 µL internal standard working stock solution (5 µg/mL oxazepam-$d_5$) to each tube.

10.6.11.5 Add 1 mL saturated borate buffer and 3 mL toluene extract solvent to each tube.

10.6.11.6 Cap and rotate tubes for 30 minutes.

10.6.11.7 Centrifuge at approx 2500 rpm for 15 minutes. Transfer upper (organic) layer to labeled conical bottom tubes. Evaporate samples to dryness under nitrogen at 85° C (approximately 1 hr).

10.6.11.8 Cool samples to room temperature. Add 50 µL MTBSTFA to each sample. Cap and heat samples at 85° C for 1 hour.

10.6.11.9 Cool samples to room temperature. Add 0.5 mL dry toluene to each sample. Vortex briefly. Transfer a small amount of each sample to labeled GC autosampler vial.

10.6.11.10 Transfer GC autosampler vials to GC/ECD and analyze. Calculate the concentrations by interpolation of a linear plot of the response curve based on peak height or area.

10.6.11.11 For any cases positive for lorazepam and/or temazepam, transfer GC vials to GC equipped with MSD for confirmation.

10.6.12 For cases in which chlordiazepoxide or n-desalkylflurazepam were detected by GC/ECD or suspected, the samples must be reanalyzed with full calibration curve for chlordiazepoxide and n-desalkylflurazepam.

10.6.12.1 Label clean 13 x 100 mm screw cap tubes accordingly, negative, calibrators, control(s) and case sample IDs.

10.6.12.2 Prepare calibrators and controls.

10.6.12.3 Pipet 1 mL of each case sample into appropriately labeled tubes.

10.6.12.4 Add 30 µL internal standard working stock solution (20 µg/mL prazepam) to each tube.

10.6.12.5 Add 1 mL saturated borate buffer and 3 mL extract solvent (78:20:2 THIA) to each tube.

10.6.12.6 Cap and rotate tubes for 30 minutes.

10.6.12.7 Centrifuge at approx 2500 rpm for 15 minutes. Transfer a small amount of upper (organic) layer to GC autosampler vial. Crimp autosampler vials and set aside for GC/ECD analysis.

10.6.12.8 Transfer remaining organic upper layer (THIA) to appropriately labeled conical bottom test tubes. Cap tubes and store in refrigerator for GC/MS confirmation of any positive samples.

10.6.12.9 Transfer GC autosampler vials to GC/ECD and analyze. Calculate the concentrations by interpolation of a linear plot of the response curve based on peak height or area. For cases in which chlordiazepoxide and/or n-desalkylflurazepam were detected by GC/ECD, remove the remaining THIA extracts from the refrigerator. Evaporate THIA extracts to dryness under nitrogen at 55° C or less.

10.6.12.9.1 Cool samples to room temperature. Add 0.5 mL acetonitrile and 2 mL hexane to each sample.

10.6.12.9.2 Vortex each sample for 30 seconds at the highest setting.

10.6.12.9.3 Aspirate off top organic layer (hexane) and discard. Evaporate remaining lower (acetonitrile) layer under nitrogen at 55° C or less.

10.6.12.9.4 Reconstitute samples with 30 µL 78:20:2 THIA and transfer to GC autosampler vials. Inject 1-2 µL of each sample onto GC equipped with MSD for confirmation.

**10.7 Calculation**

10.7.1 Calculate the concentrations by interpolation of a linear plot of the response curve based on peak height (or area) ratios versus calibrator concentration.

**10.8 Quality Control And Reporting**

10.8.1 See Toxicology Quality Guidelines

**10.9 References**

10.9.1 King, J.W. and King, L.J., Solid-phase extraction and on-disc derivatization of the major benzodiazepines in urine using enzyme hydrolysis and Toxi-Lab VC MP3 column, J. Anal. Tox 20: 262-265, 1996.

10.9.2 Flammia, D., Woods, T. and Edwards, R., in house development.

10.9.3 Mozayani, A., in house development.